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Nedd4 Branches Out

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The branching of dendrites and axons is a key determinant of neural circuit formation. In this issue of *Neuron*, Kawabe et al. demonstrate that the ubiquitin ligase Nedd4 promotes the branching of developing dendrites by targeting the small G protein Rap2. In a complementary study, Drinjakovic et al. show that Nedd4 promotes the branching of developing axons by ubiquitinating a different target, the phosphatase PTEN.

The arborization of neurites is a key step in the development and refinement of neural circuits. Developing axons and dendrites branch as synaptic contacts form. The pattern and extent of branching is a key determinant of both the number of synaptic partners made by a neuron as well as the number of synaptic connections made with each partner. As such, neurite branching affects both the wiring diagram and strength of a developing synaptic circuit. This central role for neurite branching motivates efforts to understand the molecular mechanisms at play.

Both extrinsic and intrinsic mechanisms regulate neurite branching (reviewed in Parrish et al., 2007). These include extracellular cues that likely provide spatial information for branching as well as transcriptional programs controlling cell-type-specific branching patterns. In addition, signal transduction mechanisms must link either extrinsic or intrinsic cues to pathways regulating the cytoskeletal dynamics that mediate neurite branching. While phosphorylation is likely the most common mechanism to regulate such signal transduction, work over the past decade has made clear that the covalent addition of ubiquitin to target molecules is also a powerful means to regulate pathways controlling circuit development (reviewed in Yi and Ehlers, 2007). Work in this issue of *Neuron* from the Brose and Holt labs adds to this growing body of literature, demonstrating a role for the ubiquitin ligase Nedd-4 in promoting the branching of both dendrites (Kawabe et al., 2010) and axons (Drinjakovic et al., 2010) via the inhibition of distinct molecular pathways.

Nedd4-1 encodes a ubiquitin ligase that is highly expressed in mammalian neurons. It is a member of the Nedd4 family of HECT domain E3 ubiquitin ligases. Such ligases are composed of a phospholipid-binding C2 domain that controls its intracellular targeting, a WW domain involved in substrate binding, and a HECT domain that is the catalytic portion of the protein that attaches ubiquitin to target proteins (Rotin and Kumar, 2009). Candidate substrates have been identified in a number of systems, and genetic evidence in flies and worms indicates that Nedd4 family members can regulate neural development.

To investigate the function of Nedd4-1 in the mammalian brain, Kawabe and colleagues generated knockout mice. The constitutive loss of Nedd4-1 leads to late embryonic lethality, likely due to nonneural functions. To circumvent this problem, Kawabe et al. first generated autaptic cultures of embryonic cortical neurons. They found that Nedd4-1 mutant neurons were much smaller due to a reduction in the size and complexity of their dendritic trees. They then generated a conditional knockout mouse and deleted Nedd4-1 in postmitotic glutamatergic neurons. These mice lived and revealed that dendrites of CA1 pyramidal neurons are significantly smaller and less complex in adult mice in the absence of Nedd4-1. Hence, Nedd4-1 promotes dendrite growth and branching both in vitro and in vivo.

The morphological change in the dendrite was accompanied by a functional deficit in the autaptic cultures, with mutant neurons showing a reduction in the amplitude of evoked synaptic currents. Such a defect may be due to either a decrease in the efficacy of transmission at each synapse or to a decrease in the number of synapses. There is no defect in either paired-pulse facilitation or the amplitude of miniature synaptic currents, so neither release probability nor postsynaptic sensitivity to transmitter are likely impaired. This indicates that in the absence of Nedd4-1 the synapses that form function relatively normally. Instead, counting synapses reveals an ~50% decrease compared to wild-type with no change in the density of synapses along the dendrite. These data suggest that the effect of Nedd4-1 on the synapse is secondary to its role in promoting dendrite branching.

Having found a ubiquitin ligase with an impressive neurite branching phenotype, Kawabe et al. were faced with the difficult task of identifying the substrate of the ligase. In studies of invertebrates, large-scale suppressor screens can identify the functionally relevant target (Nakata et al., 2005, Collins et al., 2006). However, in mice such an approach is not feasible. Instead, Kawabe et al. took a biochemical approach, identifying proteins in brain lysate that bind to the putative substrate-binding WW domain of Nedd4-1. Among the identified proteins, their attention turned to TINK, a kinase known to regulate actin dynamics as a downstream effector of the small G protein Rap2 (Taira et al., 2004). Indeed, coimmunoprecipitation experiments revealed that Nedd4-1, TINK, and Rap2 form a tripartite complex, with TINK serving as a linker. The formation of this complex allows Nedd4-1 to monoubiquitinate Rap2, inhibiting its function. This regulation of Rap2 function by ubiquitination is a nice example of an important but often overlooked concept—ubiquitination does more than regulate the abundance and subcellular localization of proteins, it can also regulate their activity.

While the biochemical data are beautiful, it is still essential to show that Rap2 is a functionally relevant target for the branching phenotype. Since Nedd4-1 inhibits Rap2 function, Kawabe et al. posited that in the Nedd4-1 mutant overactive Rap2 leads to the decrease in dendrite branching. To test this hypothesis, they expressed either a dominant-negative Rap2 or dominant-negative TINK and showed that either leads to an increase in dendritic complexity. Importantly, this effect was larger in the Nedd4-1 mutant than in wild-type. This differential effect on the mutant is an important indication that Nedd4-1 works via Rap2 and TINK, rather than in a parallel pathway. Also consistent with the hypothesis, overexpression of an active Rap2 or TINK gave the same phenotype as the Nedd4-1 mutant, resulting in smaller and less branched dendrites. This demonstrates that regulation of these proteins is sufficient to decrease dendrite branching. Finally, they demonstrated that ubiquitination of Rap2 is the key mechanism regulating dendrite complexity because expression of a nonubiquitinatable form of Rap2 is sufficient to inhibit dendrite branching. This work leads to a clear model of how Nedd4-1 regulates dendritic development. Nedd4-1, TINK, and Rap2 form a complex that is necessary for Nedd4-1 to monoubiquitinate Rap2. Once ubiquitinated, Rap2 can no longer activate its effector TINK. With the TINK kinase inactive, it no longer promotes dendrite retraction by regulating the actin cytoskeleton.

Working in a different system and cellular compartment, Drinjakovic and colleagues also demonstrated a role for Nedd4 in regulating neurite branching, but in this case it is branching of the axon within its target field. Previous work from the Holt lab has shown that the ubiquitin-proteasome system regulates the response of *Xenopus* retinal ganglion cell (RGC) growth cones to guidance cues such as netrin (Campbell and Holt, 2001). In order to test the role of ubiquitin-dependent regulation, they electroporated a dominant-negative ubiquitin into retinal cells of developing *Xenopus*. They observed that axon guidance proceeds normally; however, once the axon reaches its target in the tectum there is much less branching.

To investigate the mechanism of ubiquitin-dependent axonal branching, Drinjakovic et al. took a candidate approach, focusing on E3 ubiquitin ligases. They found that Nedd4 is expressed in RGC axons. To test the functional role of Nedd4, they used morpholinos as well as dominant-negative constructs to inhibit its function. Both manipulations decreased the branching of RGC axons in a manner very similar to the expression of dominant-negative ubiquitin. To identify the target of Nedd4, they again successfully used a candidate approach. In immortalized cells, Nedd4 regulates the levels of the tumor suppressor PTEN, a PIP3 phosphatase that negatively regulates the PI3K signaling pathway (Wang et al., 2008). PTEN is an interesting candidate because the PI3K pathway regulates neuronal cytoskeletal dynamics and axon turning (Cosker and Eickholt, 2007). Drinjakovic and colleagues found that Nedd4 can regulate PTEN levels in RGCs, as inhibition of Nedd4 leads to more PTEN. This regulation is functionally relevant for axonal branching. Overexpression of PTEN mimics the Nedd4 phenotype and inhibits axonal branching while knockdown of PTEN suppresses the Nedd4 phenotype, restoring axonal branching. This work leads to the model that Nedd4 ubiquitinates PTEN leading to its degradation, which would lead to an increase in PI3K signaling and axonal branching.

These two studies demonstrate a central role for the Nedd4 ubiquitin ligase in promoting neurite branching and synapse formation. While there are distinct molecular pathways in the axon and dendrite, it is striking that within each compartment the effect of losing the function of the Nedd4 E3 ubiquitin ligase appears to be primarily mediated by a single substrate. This adds to a growing literature of ligase/substrate pairs that regulate neurite branching (van Roessel et al., 2004, Nakata et al., 2005, Collins et al., 2006, Yang et al., 2010) and highlights that ligases may be specialized to target a small number of substrates and hence may provide powerful opportunities for discreet manipulations of circuit development.

The finding that ligase/substrate pairs regulate neurite branching raises two important questions. Might such developmental mechanisms be used in the mature nervous system to regulate the gain or loss of branches and hence shape circuit composition and function? If so, how might neuronal activity or extracellular cues regulate ubiquitin ligase activity? Understanding the spatial and temporal regulation of ligase function is the next big challenge in deciphering the role of ubiquitin-dependent mechanisms in shaping neural circuits.

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